Electrophoretic Studies in the Genus *Melanargia* Meigen, 1828 (Lepidoptera: Satyridae)

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Abstract. The phylogenetic relationships of the Euro-Mediterranean species of *Melanargia* were studied using enzyme electrophoresis. A dendrogram is presented representing the degree of relationship among populations sampled. Results from the biochemical characterization are only in partial agreement with those obtained from conventional systematic study.

Systematic problems at the level of the specific differentiation between *M. galathea* and *M. lachesis* are discussed on the basis of biochemical data obtained by parapatric and allopatric populations. The opinion that they represent two distinct, although recent, species is supported.

Introduction

The Satyrid genus *Melanargia* Meigen, 1828, the only genus of the subfamily Melanargiinae Wheeler, 1903, is characterized by one vein in the fore wings considerably swollen at the base. The "Marbled Whites" can be easily recognized within the family, because of distinctive characters of adult morphology (Higgins, 1975). The wings are of medium size, with creamy spots and bold black markings. The front legs are small in both sexes, with hairless femora bearing a central groove. Sexes are similar; males lack androconial scales. Male genitalia are characterized by the uncus, short, very thick at basis and swollen on the central part of ventral surface, and by the valvae, that apart from some apical spines, lack any other appendages. Female genitalia show a genital plate that is very sclerotized in front of the opening of the ductus bursae and little sclerotized posteriorly. Two small sclerotized plates are situated on either side of the external opening of the ductus bursae. The more anteriorly placed of these plates is distally swollen in a wing-shaped protuberance, which comes into contact with the sternite.

Egg morphology and larval stages are also characteristic. The eggs are oval-shaped, taller than wide, ribbed and reticulate on the chorion and with a petal-like micropylar area (Wagener, 1983). The ova are laid during flight. The night-feeding larvae are light green or sandy in colour. All feed upon Graminaceae (Higgins and Riley, 1980) and all hibernate in winter as larvae. The pupae lie free among the grass stems.

Previous taxonomic studies have concentrated on morphological relationship drawn from wing colouration and marking. The genus was divided into three subgenera: *Melanargia* Meigen, 1828, *Argeformia* Verity, 1955, and *Halimede* Oberthür & Houlbert, 1922.

The latter is a Central and East Asian group which will not be dealt with in the present paper. Of the Euro-Mediterranean species, *M.*

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galathea (Linné, 1758), *M. russiae* (Esper, 1784), *M. larissa* (Boisduval, 1828), *M. hylata* (Ménétriés, 1832), *M. titea* (Klug, 1832) and *M. syriaca* (Oberthür, 1894) were arranged in the subgenus *Melanargia* whereas *M. arge*, *M. occitanica* and *M. ines* (with its ssp. *jahandiezi* Oberthür, 1920) were arranged in the subgenus *Argeformia*. Within this same group *M. pherusa* (Boisduval, 1833) is variously considered either as a ssp. of *M. occitanica* or as a distinct species.

On the basis of external morphology, species of the subgenus *Melanargia* can be subdivided into two groups: one with *M. russiae*, *M. hylata*, *M. syriaca* and *M. larissa* and another with *M. galathea* and *M. titea*. More recently, however, Wagener (1983) suggested a different arrangement based on the ultrastructure of ova. He recognized three species groups: one including *M. galathea* and *M. syriaca*, another one with *M. larissa*, *M. titea*, *M. hylata* and the Asiatic *M. grumi* (Standfuss, 1882) and a third one with *M. russiae*, the Asiatic *M. halimede* (Ménétriés, 1858) and *M. parce* (Staudinger, 1882).

An unresolved controversy regards the relationship between *M. galathea* and *M. lachesis* (Hübner, 1790) and their dubious specific designation. The mostly parapatric distribution of *M. galathea* and *M. lachesis* in the Western and Eastern sections of the Pyrenees and the morphology of adult stages, ova and larvae have been extensively studied (Higgins, 1969; Tilley, 1983, 1986; Wagener, 1984; Mazel, 1986). Whereas in the areas of sympatry in northern Spain the two phenotypes maintain their distinctive characteristics (Gomez de Aizpúrua, 1988), in the contact zone of the Eastern Pyrenees also somewhat intermediate specimens are found, equal to one fifth of the whole mixed populations (Mazel, 1986).

Since genitalic and other structural characters are very conservative in this genus and show little, if any, variation between species, any attempt to carry out a cladistic reconstruction of its phylogeny could only be based on a very limited set of characters. The purpose of this work, therefore, is to evaluate genetic relationship among the Euro-Mediterranean species of *Melanargia* through their respective degree of electrophoretically detectable enzyme similarity. As a consequence, it also attempts to provide a yardstick for the rate of speciation processes within this genus and, at the same time, to supply new reliable taxonomic characters in the form of electromorph variants obtained by allozyme electrophoresis.

Methods and Materials

PREPARATION OF SAMPLES

Seventeen European and Turkish populations, for a total of 165 specimens, were scored for enzyme variability. Populations and localities are listed in table 1; geographical distribution of sampled localities is depicted in Fig. 1.

Only field-collected adult males were employed for enzyme analysis. Their wings were immediately removed with sharp scissors and the whole bodies were frozen in liquid nitrogen while still alive. Specimens were kept in the same medium for several days until their preparation for electrophoresis could take

Table 1. Populations sampled

Locality	Country, Region	n°	symbol	
Paola	Italy, Calabria	12	A1	(*arge*)
Fuscaldo	Italy, Calabria	3	A2	(*arge*)
Capo Cervo	Italy, Liguria	14	O1	(*occitanica*)
Mont Leuze	France, Alpes Mar.	4	O2	(*occitanica*)
Ficuzza	Italy, Sicily	10	Ph	(*pherusa*)
Melilla	Morocco	4	In	(*ines*)
Camlibel Dag.	Turkey, Sivas	10	L1	(*larissa*)
Mazikiran Gec.	Turkey, Sivas	10	L2	(*larissa*)
Onagil	Turkey, Van	7	H1	(*hylata*) *
Kocak	Turkey, Van	10	H2	(*hylata*) *
Asagi Kolbasi	Turkey, Bitlis	12	Sy	(*syriaca*)
Capo Cervo	Italy, Liguria	14	G1	(*galathea*)
Monte Amaro	Italy, Abruzzo	9	G2	(*galathea*)
Kastamonu	Turkey, Bolu	10	G3	(*galathea*)
Campo	Spain, Huesca	18	G4	(*galathea*)
Tragacete	Spain, Cuenca	10	GL1	(*lachesis*)
Campo	Spain, Huesca	8	GL2	(*lachesis*)

* These populations, once considered identical with ssp. *karabagi*, will soon be described as a distinct subspecies by S. Wagener.

Figure 1 - Geographic distribution of sampling localities of *Melanargia* populations studied. 1: Melilla; 2: Tragacete ; 3: Campo; 4: Mont Leuze; 5: Capo Cervo; 6: Monte Amaro; 7: Fuscaldo; 8: Paola; 9: Ficuzza; 10: Kastamonu; 11: Camlibel Daglari; 12: Mazikiran Gecidi; 13: Asagi Kolbasi; 14: Koçak; 14: Onagil.

place. Samples were prepared as follows. Individual butterflies were macerated, using a tissue grinder, in 300 µl of a homogenizing solution containing 0.12 mM NADP and 10mM 2-mercaptoethanol. The homogenate was centrifuged at 13000 g for 15 min to obtain a clear supernatant. Samples were stored at -80°C until electrophoresed.

ELECTROPHORESIS

Allozyme electrophoresis was carried out on Cellogel sheets at 4°C using buffer systems and stains as described by Meera Khan (1971) and Richardson et al. (1986). Thirteen gene-enzyme systems were studied: glycerol-3-phosphate dehydrogenase (E.C.1.1.1.8) (*GPD*), malate dehydrogenase (E.C.1.1.1.37) (*MDh*), malic enzyme (E.C.1.1.1.40) (*ME*), isocitrate dehydrogenases (E.C.1.1.1.42), 2 loci (*IDh-1, 2*), 6-phosphogluconate dehydrogenase (E.C.1.1.1.44) (*6PGD*), aspartate aminotransferase (E.C.2.6.1.1) (*AAt*), hexokinase (E.C.2.7.1.1) (*HK*), adenylate kinase (E.C.2.7.4.3) (*AK*), phosphoglucomutases (E.C.2.7.5.1), 2 loci (*PGM-1, 2*), mannose phosphate isomerase (E.C.5.3.1.8) (*MPI*), phosphoglucose isomerase (E.C.5.3.1.9) (*PGI*). Isozymes and alleles were designated numerically according to their decreasing mobility rate.

STATISTICAL ANALYSIS

Average probability of interpopulation genetic identity and distance were calculated by Nei's I and D related indexes (jackknifed according to Mueller and Ayala, 1982). A number of other more or less similar indexes were also computed: Rogers' distance, Wright's modification of Rogers' distance, Cavalli-Sforza's & Edwards' arc and chord distances (for these indexes see Wright, 1978) and Hillis' modification of Nei's distance (1984). Several dendrograms resulting from the

Table 2. Allele frequencies

		A1	A2	O1	O2	Ph	In	L1	L2	H1	H2	Sy	G1	G2	G3	G4	GL1	GL2
αGPD	*120*								0.05									
	100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.95	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
MDH	*120*							0.40	0.35									
	100			0.07				0.60	0.65	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	90	1.00	0.83	0.93	1.00	0.94	1.00											
	65		0.17			0.06												
ME	*120*						1.00										0.20	0.25
	110									0.21		0.10			0.05			
	100	1.00	1.00	1.00	1.00	0.61		0.75	0.15	0.43	1.00	0.90	1.00	0.78	0.95	1.00	0.75	0.75
	90					0.39		0.25	0.85	0.36				0.22			0.05	
IDH-1	110															0.14	0.28	0.25
	100	1.00	1.00	0.32	0.25	0.17	0.25		0.15		0.05	0.08	1.00	0.94	1.00	0.86	0.72	0.75
	90			0.68	0.75	0.83	0.62	1.00	0.70	1.00	0.95	0.92		0.06				
	80						0.12		0.15									
IDH-2	130	1.00	1.00															
	100			1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

		A1	A2	O1	O2	Ph	In	L1	L2	H1	H2	Sy	G1	G2	G3	G4	GL1	GL2
6PGD	120											0.29	0.07				0.17	0.25
	110							0.06		0.07		0.08	0.25	0.14	0.25	0.06	0.58	0.31
	100	0.92	1.00					0.33			0.05	0.37			0.05			
	80							0.39	0.70	0.28	0.10	0.08	0.64	0.86	0.70	0.94	0.25	0.44
	70	0.08		1.00	1.00	1.00	1.00	0.11	0.30	0.50	0.85	0.17	0.03					
	50							0.11		0.14								
AAt	150											0.04						
	140			0.03	0.12			0.55	0.70	0.07	0.15	0.42		0.11				
	120			0.11	0.25					0.43	0.65	0.33	0.28	0.11		0.10		
	110							0.05	0.10	0.14								
	100			0.32	0.37	0.33		0.40	0.05	0.28	0.10	0.17	0.53	0.55	1.00	0.60	0.44	0.44
	80	1.00	1.00	0.53	0.25	0.67	1.00		0.15	0.07	0.05	0.04	0.18	0.17		0.25	0.56	0.56
	70										0.05			0.05		0.05		
HK	120											0.04						
	110			1.00	1.00	1.00	1.00		0.10			0.08						
	100	0.83	1.00					1.00	0.90	1.00	1.00	0.87	1.00	1.00	1.00	1.00	1.00	1.00
	90	0.17																
AK	150				—	—	—						0.07	0.17		—	—	—
	120				—	—	—							0.33		—	—	—
	100	1.00	1.00	1.00	—	—	—	1.00	0.95	1.00	0.95	0.82	0.93	0.50	1.00	—	—	—
	75				—	—	—		0.05		0.05	0.18				—	—	—
PGM-1	110	—	—	—	—	—	—			0.07	0.05	0.17		0.11	0.35	0.28	0.19	0.36
	100	—	—	—	—	—	—	0.30	0.15	0.93	0.90	0.75	1.00	0.83	0.65	0.71	0.81	0.62
	90	—	—	—	—	—	—	0.70	0.85		0.05	0.08		0.06				0.02
PGM-2	100							1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	80			0.12		1.00	0.87											
	75	1.00	1.00															
	60			0.88	1.00		0.12											
MPI	150				—	—	—		0.17		0.10					—	—	—
	145				—	—	—	0.30	0.17	0.08		0.20	0.22	0.19	0.05	—	—	—
	135				—	—	—	0.20	0.33	0.08	0.10	0.50	0.28	0.44	0.11	—	—	—
	130				—	—	—	0.10		0.08	0.05		0.50	0.37	0.83	—	—	—
	120			0.18	—	—	—	0.40	0.28	0.33	0.30	0.15				—	—	—
	110			0.32	—	—	—									—	—	—
	100	0.96	0.83	0.14	—	—	—		0.05	0.33	0.10	0.15				—	—	—
	90	0.04	0.17	0.25	—	—	—			0.08	0.35					—	—	—
	80			0.11	—	—	—									—	—	—
PGI	175												0.03					
	160											0.12	0.03	0.33	0.05			
	145			0.73	0.70			0.45	0.14			0.54	0.11	0.39	0.25		0.37	0.62
	100	1.00	1.00	0.10	0.20	1.00		0.45	0.28	0.10	0.12	0.17	0.14	0.11	0.70	0.56	0.44	0.31
	75			0.10				0.10	0.57	0.60	0.75	0.17	0.53	0.11		0.12	0.19	0.06
	60						1.00									0.31		
	55			0.07														
	40									0.30	0.12		0.14	0.06				

— indicates that in the population scoring was not possible.
For some enzymes not all individuals of all populations were scored.

Table 3 Jackknifed average Nei Distances between populations

	A1	A2	O1	O2	Ph	In	L1	L2	H1	H2	Sy	G1	G2	G3	G4	GL1	GL2
A1	—	0.00	0.07	0.11	0.09	0.21	0.10	0.13	0.11	0.12	0.08	0.10	0.10	0.09	0.09	0.07	0.08
A2	0.01	—	0.07	0.12	0.10	0.22	0.10	0.13	0.12	0.12	0.08	0.09	0.10	0.09	0.09	0.07	0.08
O1	0.68	0.69	—	0.00	0.02	0.04	0.05	0.06	0.04	0.04	0.05	0.08	0.07	0.08	0.11	0.08	0.08
O2	0.81	0.84	0.02	—	0.02	0.05	0.06	0.09	0.07	0.07	0.05	0.11	0.09	0.10	0.11	0.09	0.09
Ph	0.64	0.66	0.20	0.23	—	0.03	0.08	0.07	0.08	0.09	0.09	0.12	0.13	0.11	0.10	0.10	0.11
In	0.96	0.99	0.33	0.39	0.23	—	0.26	0.16	0.16	0.17	0.20	0.29	0.29	0.43	0.18	0.18	0.17
L1	0.77	0.77	0.58	0.55	0.63	1.04	—	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.02	0.02	0.02
L2	1.00	1.00	0.72	0.78	0.69	0.92	0.13	—	0.01	0.02	0.01	0.02	0.02	0.03	0.03	0.03	0.03
H1	0.88	0.88	0.55	0.58	0.61	0.87	0.26	0.24	—	0.00	0.01	0.01	0.02	0.02	0.02	0.02	0.03
H2	0.89	0.88	0.49	0.53	0.62	0.83	0.29	0.31	0.07	—	0.01	0.02	0.02	0.03	0.03	0.03	0.04
Sy	0.76	0.76	0.53	0.48	0.71	0.97	0.15	0.28	0.20	0.18	—	0.01	0.01	0.02	0.03	0.02	0.02
G1	0.73	0.73	0.71	0.73	0.86	1.18	0.32	0.38	0.23	0.26	0.27	—	0.00	0.00	0.00	0.00	0.00
G2	0.78	0.78	0.69	0.65	0.83	1.18	0.28	0.38	0.31	0.37	0.23	0.07	—	0.00	0.00	0.00	0.00
G3	0.65	0.65	0.74	0.72	0.76	1.25	0.29	0.48	0.36	0.41	0.33	0.09	0.10	—	0.00	0.00	0.00
G4	0.61	0.61	0.75	0.75	0.72	1.05	0.30	0.43	0.30	0.33	0.32	0.07	0.08	0.03	—	0.00	0.00
GL1	0.58	0.58	0.61	0.67	0.70	1.00	0.32	0.47	0.33	0.35	0.25	0.10	0.11	0.09	0.10	—	0.00
GL2	0.63	0.62	0.58	0.64	0.76	0.98	0.31	0.47	0.36	0.39	0.24	0.12	0.08	0.08	0.10	0.03	—

Below the diagonal values of distances, associated standard errors above.

UPGMA and Fitch-Margoliash (1967) clustering of all such indexes were also constructed.

Results

Allele frequencies are shown in Table 2. The overall number of alleles detected at the 13 loci of all *Melanargia* species studied amounts to 61 (mean per locus 4.69, range 2-9). No locus proved monomorphic across the whole sample.

The jackknifed Nei's indexes of genetic similarity, I, and genetic distance, D, were calculated on the basis of the 13 shared loci for pairwise combinations of all populations studied (table 3). A dendrogram representing the degree of relationships between populations sampled is depicted in fig. 2 for Nei's indexes.

Rogers' index, Wright's modification of the same, Cavalli-Sforza's arch and chord measures, and Hillis' average distances were also computed (data not shown) and employed to generate clusters, that proved almost identical with the dendrogram shown in fig. 2.

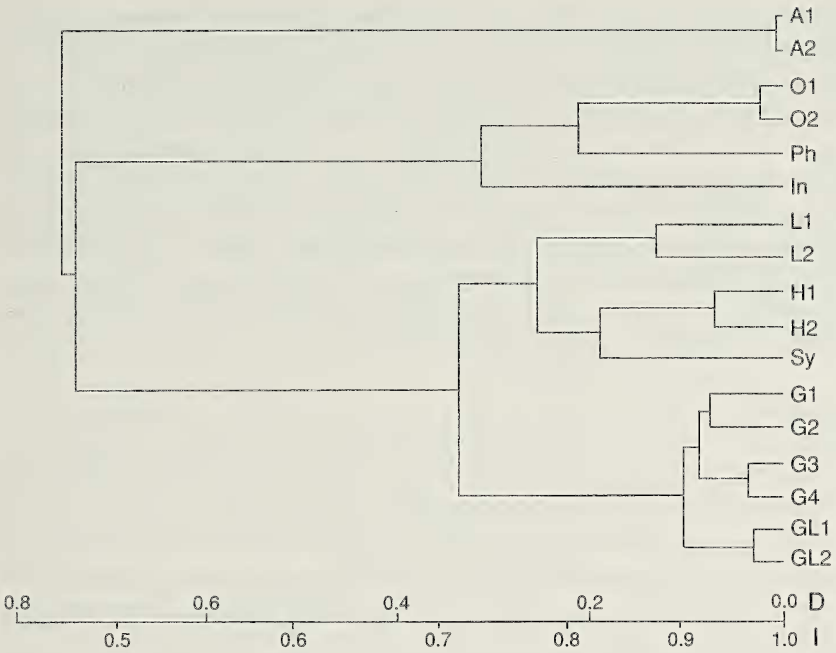

Figure 2 - Dendrogram based on jackknifed Nei's identities

Species fall clearly into four groups, as follows:
1) *Melanargia arge*
2) *M. occitanica, M. pherusa* and *M. ines*
3) *M. larissa, M. hylata* and *M. syriaca*
4) *M. galathea* and *M. lachesis*

The populations of *M. arge* are the most differentiated with respect to all other populations studied (D = 0.753, with respect to others). Rather surprisingly, *M. arge* shows complete fixation at 8 loci (61% of total). Remarkably, 2 of these (PGM-2 and IDh-2) proved alternative with respect to the other *Melanargia* studied.

In contrast, no private locus was found to characterizes group (2). Within this group *M. occitanica* and *M. pherusa* are more closely related to each other (D = 0.215) than they are with respect to *M. ines* (D = 0.314). This relatively higher value of D agrees with the traditional separation between *M. occitanica* and *M. ines*: in this case genetic divergence correlates with morphological and biological differences (in fact in Spain *M. occitanica* and *M. ines* fly together without interbreeding).

Groups (3) and (4), corresponding to subgenus *Melanargia*, cluster at a value of D = 0.334 and share a diagnostic locus (at PGM2 they fix allele *100*, absent in other groups).

Within group (3) the closest electrophoretic similarity is shown by the two populations of *M. hylata*.

Interestingly enough, electromorph variation ranks *M. syriaca* within species of group 3, whereas on the basis of its egg morphology (Wagener, 1983), it should be expected to be more similar to *M. galathea*.

A high degree of genetic similarity is demonstrated by the analysis of group 4, even between geographically very distant populations: for example D values between the Turkish and the Spanish sample of *M. galathea* (0.032) is even lower than that found between the Italian populations of Capo Cervo (N.W. Italy) and Monte Amaro (C. Italy) (D = 0.074). Even though these values appear very low, it may be relevant to observe that both were proved to differ significantly from zero by the jackknife procedure (Tab. 3 and Fig. 2).

Discussion

Results from the biochemical characterization of entities belonging to the genus *Melanargia* is only in partial agreement with those obtained from other more conventional systematic studies. The main branching point of the dendrogram shown in Fig. 2 is between *M. arge* and the other groups of *Melanargia* studied (D = 0.753). This accounts for the isolated position of the populations of *M. arge* in the dendrogram. However, the group including *M. occitanica*, *M. pherusa* and *M. ines* branches at D = 0.739, which is only slightly below the highest level of genetic distance. Therefore these biochemical-genetic criteria support a taxonomical arrangement different from the current subgeneric differentiation of *Melanargia* and *Argeformia*.

Since *M. arge* proved highly monomorphic over the whole sample of enzymes investigated (H = 0.039), values for average heterozygosity vary depending on whether or not data for *M. arge* are included (H = 0.124 with, or H = 0.210 without *M. arge*). The first of these values is in better agreement with average values found for a number of invertebrate species (H = 0.134, Ayala et al., 1972). The exceedingly low heterozygosity of *M. arge* could be the effect of a recent bottle-neck. This species, in fact, may well have survived glaciations even in a single refuge area in the South of the Italian peninsula (or perhaps in Sicily), where it remained restricted at least since the last ice age.

Genetic differences between *M. galathea* and *M. lachesis* are relatively small, but within the range previously reported for closely related species of Lepidoptera (Geiger, 1988).

External (phenetic and morphological) characteristics of the two forms are notably different, *M. lachesis* being larger and with reduced black markings on the dorsal surface of the wings. Their distribution is broadly parapatric and, apart from a few small areas of sympatry, in the Iberian Peninsula their biotopes are sharply delineated by altitude and exposure. *M. galathea* is a sub-nemoral species (Balletto and Kudrna, 1985) diffused throughout Europe. Its typical biotope is in the outskirts, or the clearings of, mesophilous woods, at elevations generally not exceeding 800-900 m, where the vegetation includes an abundant growth of *Phleum*

pratense, the main foodplant of its larvae. In SE France and the southern belt of the Pyrenees *M. galathea* is replaced by *M. lachesis*. The latter species flies generally in dry and warm biotopes with a vegetation of a Mediterranean or sub-Mediterranean type, where its larval food plant, *Lamarkia aurea*, grows.

Notwithstanding their somewhat different environmental and climatic requirements, both species are known to live in sympatry in some fifteen biotopes of the antipyrenaic Spanish districts of Huesca, Lerida and Gerona and in only one biotope in Southern France (Col de Grès, in the Aude; Mazel, 1986), between the mouth of the River Rhone and the Pyrenees. In either case, the areas of sympatry are extremely small, in relation at least to the extension of the parapatric populations.

Interestingly, in the contact area of southern France some roughly intermediate individuals have been described, which up until now remain unreported from the Spanish side of the Pyrenees. Even though this was not experimentally demonstrated, such specimens are often supposed to be hybrids (Higgins, 1969; Mazel, 1986).

We studied two sympatric populations at a single Spanish biotope, whereas the French zone of sympatry between the two species could not yet be investigated. We found only typical individuals, easily classifiable either as *M. galathea* or *M. lachesis*. Data reveal that these sympatric populations are not significantly more similar to each other (D = 0.098) than to allopatric samples (D = 0.103), although in any case no diagnostic locus could be found. This finding suggests that little, if any, introgression between the two species may exist, and is in agreement with the hypothesis that there is a separation at species level between *M. lachesis* and *M. galathea*.

A rough dating of reproductive isolation between the two taxa is provided by time divergence estimates based on D values. Nei's estimates (Nei, 1972) to match electrophoretic data to the molecular clock hypothesis have been much questioned (see Mindell et al., 1990); we tentatively apply Nei's calibration, as it has often been shown to match very well with current geological and biogeographical knowledge (Mensi et al., 1987).

Based on these estimates, in the evolutionary history of genus *Melanargia*, *M. galathea* and *M. lachesis* split roughly 0.5 Myr ago. In the light of such a recent separation, it is not surprising that in nature they may perhaps be still capable of producing hybrids, although exceptionally.

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